REMARKS

Summary of the Invention

The present invention features the discovery of the mammalian methionine synthase reductase gene. The methionine synthase reductase gene encodes a protein which catalyzes the reductive methylation of methionine synthase-cob(II)alamin to generate methionine synthase-cob(III)alamin-CH3, thereby maintaining methionine synthase in its reduced, activated state. Mutations in the methionine synthase reductase gene have been discovered to be associated with neural tube defects, cardiovascular disease, and cancer. The invention provides wild-type and mutant mammalian methionine synthase reductase nucleic acid molecules and their complements.

Summary of the Telephonic Interview

Applicants wish to thank the Examiner and the Examiner's supervisor for the telephonic interview conducted on September 7, 2004. During the telephonic interview, Applicants discussed the 35 U.S.C. § 112 first and second paragraph rejections of claims 1-2, 4-5, 36-37, 41-43, 45-47, and 52-53, and claims 5, 41-43, 45-46, 52-55, respectively. A discussion of each of the interview topics is provided below.

Summary of the Office Action

Claims 1-5, 36-38, 41-43, 45-49, and 52-55 are pending. Claims 48 and 49 are withdrawn from consideration as being drawn to a non-elected invention. Claims 5, 41-43, 45-46, and 52-55 are rejected under 35 U.S.C. § 112, second paragraph, for indefiniteness, while

claims 1-2, 4-5, 36-37, 41-43, 45-47, and 52-53 are rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. By this reply, Applicants cancel claims 1-5, 36-38, 41-43, 45-47, and 52-55, add new claims 56-75, and address each of the Examiner's rejections.

Support for the Amendment

Support for new claims 56-75 is found in prior claims 1-5, 36-38, 41-43, 45-47, and 52-55 and in the specification on, e.g., page 5, line 5, through page 6, line 11, page 7, lines 7-17, page 13, line 18, through page 14, line 16, page 16, lines 2-10, page 18, lines 9-17, and page 22, lines 14-18. No new matter is added by the amendment. Applicants provide the following table of correspondence to show the Examiner where support for each claim is derived.

Table of Correspondence

Claim	Support
56	Prior claims 1, 3, and 36-38; page 16, lines 2-10; and page 18, lines 9-17, of the specification
57	Prior claims 1, 3, and 36-38; page 5, lines 5-11, of the specification
58	Prior claims 1, 2, 3, and 36-38; page 5, lines 5-11, of the specification
59	Prior claims 1, 36, and 45; page 16, lines 2-10, of the specification
60	Prior claims 1, 36, 45, and 46; page 16, lines 2-10, of the specification
61	Prior claims 1, 36, and 47-49
62	Prior claims 36 and 54
63	Prior claim 4; page 22, line 14-18, of the specification
64	Prior claim 5
65	Prior claims 41-43; page 5, lines 15-25; page 17, line 6, through page 18, line 1; page 18, lines 9-17; and page 22, lines 14-18, of the
66	Prior claims 41-43, page 5, lines 15-25; page 17, line 6, through page 18, line 1; page 18, lines 9-17; and page 22, lines 14-18, of the specification
67	Prior claim 52
68	Prior claims 53 and 55
69	Prior claims 3 and 36-38; page 7, lines 10-17; page 16, lines 2-10; and page 18, lines 9-17, of the specification
70	Prior claims 3 and 36-38; page 7, lines 10-17, of the specification

71	Prior claims 36 and 54
72	Prior claim 1; page 16, lines 2-10; page 18, lines 9-17; page 40, line 12, through page 41, line 20; and page 42, line 6, through page 44, line 14; page 51, line 23, through page 54, line 4, of the specification
73	Prior claims 1 and 41-43; page 5, line 5, through page 6, line 11; page 18, lines 9-17, and page 22, lines 14-18, of the specification
74	Prior claims 1, 36, and 47-49
75	Prior claims 36 and 54

Rejections under 35 U.S.C. § 112, second paragraph

Claims 5, 41-43, and 52-53, and 55

Claims 5, 41-43, 45-46, and 52-55 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failure to distinctly claim the subject matter which Applicants regard as the invention. The Examiner states that claims 5, 41-43, and 52-53, and 55 "are indefinite in the recitation of 'complementary' and 'complement' because it is unclear which 'complements' are encompassed by the claims. Applicants have not define[d] the term 'complement', as is relates to size..." (Office Action, p. 2).

During the telephonic interview, the Examiner indicated that this rejection could be overcome by replacing the term "complementary" with "completely complementary" to clarify that the recited nucleic acid sequence was complementary over the entire sequence of SEQ ID NO: 1, 41, 43, 45, or 47. In response, Applicants have cancelled claims 5, 41-43, 52-53, and 55, and have provided new claims 63-68 and 73-75, which are directed to an antisense nucleic acid molecule in which the nucleic acid sequence is "completely complementary" across the indicated sequence of SEQ ID NOs: 1, 41, 43, 45, or 47. Based on the Examiner's remarks in the Office Action and during the telephonic interview of September 7, 2004, Applicants believe that new claims 63-68 and 73 do not lack clarity with respect to which "complements" are encompassed

by the claims. Therefore, this rejection should be withdrawn and should not be applied to new claims 63-68 and 73-75.

Claims 45 and 46

Claims 45 and 46 are rejected for reciting the term "biological activity" without defining that activity in the claims. In the Office Action, the Examiner suggests amending the claims to recite that the nucleic acid molecule encodes a "mammalian methionine synthase reductase polypeptide having at least [X]% of the methionine synthase reductase activity of the methionine synthase reductase polypeptide of SEQ ID NO: 2" (Office Action, p. 3; Emphasis added). During the telephonic interview, the Examiner confirmed that inclusion of this clear definition for the intended biological function would overcome this rejection. Therefore, new claims 59 and 60, which correspond to cancelled claims 45 and 46, respectively, have substituted the term "biological activity" with "at least 20%" and "at least 55%" "methionine synthase reductase activity," respectively. Accordingly, Applicants respectfully request that the rejection of claims 45 and 46 be withdrawn and should not be applied to new claims 59 and 60.

Claims 53 and 55

Claims 53 and 55 are rejected for indefiniteness for reciting a nucleic acid molecule having a polynucleotide sequence the complement of which comprises "a naturally-occurring mammalian methionine synthase reductase mutation or polymorphism." The Examiner states that claims 53 and 55 do not require that the nucleic acid molecule is a mammalian methionine synthase reductase gene or that it has the function of a polypeptide encoded by such a gene. For

this reason, the Examiner argues that it is unclear how the term further limits the claims. The Examiner notes that the rejection of claims 53 and 55 was not applied to claim 54.

Applicants have cancelled claims 53 and 55, and now provide new claim 68, which recites that the antisense nucleic acid molecule comprises a polynucleotide sequence the complete complement of which comprises a naturally-occurring mutation or polymorphism present in a mammalian methionine synthase reductase gene. Accordingly, new claim 68 clarifies that the mutation or polymorphism is one that is found in a mammalian methionine synthase reductase gene in nature and is not a mutation or polymorphism that is produced by artificial methods. Therefore, the rejection of claims 53 and 55 should be withdrawn and should not be applied to new claim 68.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 1-2, 4-5, 36-37, 41-43, 45-47, and 52-53 are rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. In the present Office Action, the Examiner states that the specification is enabling for claims 3 and 38, which are directed to a nucleic acid molecule that has 100% and 95% sequence identity to SEQ ID NO: 1, 41, 43, 45, or 47, respectively, but that the specification is not enabling for a nucleic acid molecule that has 50%, 85%, or 90% sequence identity to SEQ ID NO: 1, 41, 43, 45, or 47, or to the complement thereof, as is recited in claims 1-2, 4-5, 36-37, 41-43, 45-47, and 52-53 (Office Action pp. 4 and 7). The Examiner reiterated this position during the telephonic interview of September 7, 2004. Applicants respectfully disagree, but in an effort to expedite prosecution of the application, Applicants have cancelled claims 1-5, 36-38, 41-43, 45-47, and 52-53 and now provide new claims 56-58 and 69-70, which

correspond to prior claims 3 and 38 and which were deemed allowable by the Examiner in the Office Action and during the telephonic interview of September 7, 2004. Because claims 56-58 and 69-70 are directed to subject matter that the Examiner has deemed allowable, Applicants respectfully request that the rejection of claims 1-2, 4-5, 36-37, 41-43, 45-47, and 52-53 under 35 U.S.C. § 112, first paragraph, should not be applied to new claims 56-58 and 69-70.

Applicants also provide new claims 59-62 and 71, which depend from new claim 56 and 69, respectively, and which correspond to prior claims 45-47 and 52. Claims 59 and 60 are directed to a nucleic acid molecule with at least 95% sequence to SEQ ID NO: 1 and that encodes a mammalian methionine synthase reductase polypeptide that has at least 20% (claim 59) or 55% (claim 60) of the methionine synthase reductase activity of the methionine synthase reductase polypeptide of SEQ ID NO: 2. Claim 61 recites that the nucleic acid molecule of claim 56 further comprises a consensus binding site for one or more cofactors selected from the group consisting of FAD, FMN, and NADPH, in which the sequence of the binding site is selected from any one of SEQ ID NOs: 25 or 52-61. As discussed during the telephonic interview of September 7, 2004, Applicants have limited the sequences recited in claim 61 to those that correspond to cofactor binding sites that are naturally present in the methionine synthase reductase polypeptide encoded by SEQ ID NO: 1. Finally, claims 62 and 71 specify that the nucleic acid molecule of claim 56 and 69, respectively, comprises a naturally-occurring mutation or polymorphism present in a mammalian methionine synthase reductase gene.

Because claims 59-62 and 71 depend from, and further limit the subject matter of, independent claims 56 and 69, respectively, which the Examiner has deemed to contain allowable subject matter, Applicants respectfully submit that dependent claims 59-62 and 71 are

also in condition for allowance, and respectfully request that the rejection of claims 45-47, and 52 not be applied to new claims 59-62 and 71.

The Examiner also states that the specification is not enabling for a nucleic acid molecule that hybridizes under specific conditions to the polynucleotides of SEQ ID NO: 1, 41, 43, 45, or 47, or that corresponds to the complete complement of a polynucleotide sequence having at least 50%, 85%, or 95% sequence identity to SEQ ID NOs: 1, 41, 43, 45, or 47, where the nucleic acid molecule is capable of reducing the expression of methionine synthase reductase polypeptide, as is recited in claims 4, 5, 41-43, 52, and 53 (Office Action, p. 4). Applicants respectfully disagree, but have cancelled claims 4, 5, 41-43, 52, and 53 and provide new claims 63-68, which are directed to an antisense nucleic acid molecule that hybridizes in 2x SSC medium at 40°C to the polynucleotides of SEQ ID NOs: 1, 41, 43, 45, or 47 (independent claim 63) or that has a polynucleotide sequence that is completely complementary to a polynucleotide sequence having at least 95% sequence identity to at least 18 continguous nucleotides of SEQ ID NOs: 1, 41, 43, 45, or 47 (independent claim 65), in which the nucleic acid molecule decreases the expression of methionine synthase reductase polypeptide.

Present claims 63-68 are enabled by the specification. The specification discloses and enables antisense nucleic acid molecules that can be administered therapeutically to a mammal as an inhibitor to promote a decrease in the activity of a methionine synthase reductase polypeptide encoded by a methionine synthase reductase gene (see, e.g., page 10, lines 5-12, and page 22, lines 14-18, of the specification). As was discussed in the previous Reply to Office Action, filed on March 2, 2004, the specification provides considerable guidance for determining whether an antisense molecule is capable of causing a decrease in the expression of a methionine synthase

reductase polypeptide. For example, at page 35, line 15, through page 36, line 20, the specification teaches the use of an enzyme-linked immunosorbant assay (ELISA) for determining whether a compound (e.g., an antisense nucleic acid molecule) is able to modulate the level of expression of a methionine synthase reductase polypeptide. Page 37, lines 1-18, of the specification describes the use of a quantitative PCR assay for determining the ability of a compound to modulate the level of a methionine synthase reductase polypeptide (i.e., by detecting the amount of mRNA levels in a sample). Therefore, the specification provides the skilled artisan with sufficient guidance to make and use the invention recited in claims 63-68.

Furthermore, as is discussed above, the Examiner concedes that the specification is enabling for nucleic acid molecules having at least 95% sequence identity to the polynucleotide sequence of SEQ ID NOs: 1, 41, 43, 45, or 47. It is well within the purview of one skilled in the art to prepare antisense molecules that are complementary to such nucleic acid molecules as this technology was well known in the art prior to Applicants' priority date of January 16, 1998 and would require nothing more than routine experimentation (see, e.g., Moroni et al., J. Biol. Chem. 267:2714-2722, 1992; a copy of which is provided as Exhibit A). Therefore, because the specification enables any person skilled in the art to make "sense" strand nucleic acid molecule, the preparation of complementary "antisense" molecules recited in claims 63-68 must also be enabled by the specification. Accordingly, the rejection of claims 45-47, and 52 under 35 U.S.C. § 112, first paragraph, for lack of enablement should be withdrawn and should not be applied to new claims 63-68.

Finally, Applicants have added new claims 72-75. Claim 72 is directed to a nucleic acid molecule that is prepared by obtaining and purifying a naturally-occurring nucleic acid molecule

from a mammalian cell, in which the nucleic acid molecule has at least 90% sequence identity to SEQ ID NO: 1 and encodes a polypeptide that has methionine synthase reductase catalytic activity. Claim 73 is directed to an antisense nucleic acid molecule that is prepared by obtaining a nucleic acid molecule from a mammalian cell, in which the nucleic acid molecule has at least 90% sequence identity to SEQ ID NO: 1, and reverse-transcribing the nucleic acid molecule to obtain an antisense nucleic acid molecule that has a polynucleotide sequence that is completely complementary to a polynucleotide sequence having at least 95% sequence identity to at least 18 contiguous nucleotides of SEQ ID NO: 1, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, or SEQ ID NO: 47 and that is capable of decreasing the expression of methionine synthase reductase. Claims 74 and 75, which depend from claims 72 and 73, specify that the nucleic acid molecule encodes a polypeptide that comprises one or more FAD, FMN, or NADPH cofactor binding sites and that the nucleic acid molecule comprises a naturally-occurring mutation or polymorphism present in a mammalian methionine synthase reductase gene, respectively. As discussed during the telephonic interview with respect to the subject matter of new claim 72, undue experimentation would not be required to identify nucleic acid molecules having 90% percent sequence identity to SEQ ID NOs: 1, 41, 43, 45, or 47 that fall within the scope of claim 72 because the nucleic acid molecules that are the product of the method are obtained directly from, and are naturally present within, mammalian cells. Furthermore, new claim 72 recites that the nucleic acid molecules include only those that encode methionine synthase reductase polypeptides having naturally-occurring mutations or polymorphisms present in a mammalian methionine synthase reductase gene. Therefore, claim 72 does not include methionine synthase reductase nucleic acid molecules that contain artificially generated mutations. In addition, claim

72 further requires that the nucleic acid molecule encode a polypeptide with methionine synthase reductase biological activity (i.e., the ability to catalyze the reductive methylation of methionine synthase-cob(II)alamin to generate methionine synthase-cob(III)alamin-CH3). The specification provides considerable guidance with respect to the identification of such nucleic acid molecules and the testing of polypeptides encoded by the nucleic acid molecules for methionine synthase reductase biological activity (see, e.g., page 35, lines 11-14, and page 46, line 5, through page 54, line 4). Therefore, claim 72 is enabled to the full scope and the rejection of claims 1-2, 4-5, 36-37, 41-43, 45-47, and 52-53 should not be applied to new claim 72, or to claims 74 and 75 which depend from claim 72.

Similarly, claim 73 is also enabled by the specification to its full scope. As is discussed above, the specification enables a method that results in the isolation, from a mammalian cell, of a nucleic acid molecule having at least 90% sequence identity to SEQ ID NO: 1 and encoding a polypeptide that has methionine synthase reductase biological activity. The method of claim 73 further involves reverse-transcribing all or a portion of that nucleic acid molecule to prepare a completely complementary nucleic acid molecule in which at least 18 contiguous nucleotides share at least 95% sequence identity with the sequence of SEQ ID NO: 1, 41, 43, 45, or 47. Because the nucleic acid molecules prepared by the method of claim 73 are derived from naturally-occurring mammalian methionine synthase reductase genes, the antisense nucleic acid molecules that are the products of the method of claim 73 include naturally-occurring mutations and polymorphisms. Furthermore, as is discussed above, the preparation of complementary "antisense" nucleic acid molecules from "sense" nucleic acid molecules was well within the purview of the skilled artisan at the time the application was filed (see Moroni et al., *supra*).

Therefore, claim 73 is also enabled to the full scope and the rejection of claims 1-2, 4-5, 36-37, 41-43, 45-47, and 52-53 should not be applied to new claim 73, or to claims 74 and 75 which depend from claim 73.

For all of the reasons discussed above, the specification provides considerable enabling guidance to the skilled artisan for practicing the full scope of new claims 56-75. Therefore, Applicants respectfully request that the rejection of claims 1-2, 4-5, 36-37, 41-43, 45-47, and 52-53 under 35 U.S.C. § 112, first paragraph, should be withdrawn and should not be applied to new claims 56-75.

CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is respectfully requested. Enclosed is a petition to extend the period for replying for two months, to and including October 19, 2004.

If there are any charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 19 October 2004

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

Exhibit A

Vol. 267, No. 5, Issue of February 5, pp. 2714-2722, 1992
Printed in U.S.A.

EGF-R Antisense RNA Blocks Expression of the Epidermal Growth Factor Receptor and Suppresses the Transforming Phenotype of a Human Carcinoma Cell Line*

(Received for publication, June 24, 1991)

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We have used an antisense approach to investigate the role of overexpression of the normal human epidermal growth factor (EGF) receptor in the transformed phenotype of KB cells, which are a tumor derived human cell line.

Initial experiments performed *in vitro*, showed that antisense RNA complementary to the entire coding region (AS-FL) or to parts of the EGF-R mRNA (AS-3′, AS-5′, and AS-K) effectively blocked translation of EGF-R mRNA. In addition, upon microinjection into KB cells, the *in vitro* synthesized antisense RNAs were able to inhibit transiently the synthesis of EGF-R. Inhibition was concentration-dependent, both *in vitro* and in cells, and the most effective constructs were those complementary to the entire coding region (AS-FL) or to the 3′-coding end of the mRNA (AS-3′).

Transfection of the same EGF-R antisense RNA constructs into the human epidermoid carcinoma KB cell line gave rise to several clones stably expressing elevated levels of antisense RNA and resulting in low residual levels of EGF receptor. The most reduced clones exhibited a totally restored serum-dependent growth and were severely impaired in colony formation and growth in agar. In addition the severity of the phenotype was directly proportional to the residual amount of EGF-R expressed. We conclude that overexpression of normal EGF-R plays a direct primary role in the development of the transformed phenotype of this human cancer cell line.

Epidermal growth factor is a potent mitogen controlling the growth of various types of cells through its specific receptor (Carpenter and Cohen, 1990). The epidermal growth factor receptor (EGF-R)[1] has an intrinsic tyrosine kinase activity and upon ligand activation is able to phosphorylate itself and cellular substrates on tyrosine (for review see Hunter, 1991). Phosphorylation initiates a series of events eventually resulting in cell division and proliferation.

In addition to serving a normal physiological function, the EGF-R has been consistently linked to transformation, both in model systems and in human tumors. The transforming gene product of the avian erythroblastosis virus, v-*erb* B, is homologous to the truncated chicken EGF-R but lacks its extracellular domain (Ullrich *et al.*, 1984; Lax *et al.*, 1988). The EGF-R gene is consistently amplified and overexpressed in human tumors of ectodermal origin (glioblastomas, squamous carcinoma of the lung, mammary gland, and skin) (Libermann *et al.*, 1985; Veale *et al.*, 1987; Nicholson *et al.*, 1988; Yamamoto *et al.*, 1986), suggesting an important role of the normal receptor in the development of these neoplasia. In addition we (Velu *et al.*, 1987, 1989a, 1989b) and others (Di Fiore *et al.*, 1987; Haley *et al.*, 1989; Riedel *et al.*, 1988) have shown that overexpression of the normal human EGF-R is able to transform fibroblasts in culture and induce tumors in animals. However oncogenic activity is dependent on the presence of one of its ligands, EGF or TGFα, indicating that the activated normal EGF-R is sufficient to induce a fully transformed phenotype.

Although few attempts have been made to reduce EGF-R expression in human cell lines (Buss *et al.*, 1982; Amano *et al.*, 1988; Aboud-Pirak *et al.*, 1988), the causal role played by the EGF-R in the development of human tumors has yet to be defined. EGF-R overexpression could be a primary event in inducing transformation or a secondary modification that facilitates growth and transformation of human cells.

In several cases the presence of multiple mutations has been detected in primary human tumors, including activation of proto-oncogenes and deletion or mutation of anti-oncogenes (for reviews Hunter, 1991; Marshall, 1991). In recent reports it has been shown that expression of the wild type anti-oncogene (Baker *et al.*, 1990; Bookstein *et al.*, 1990) or re-introduction of the normal chromosome (Tanaka *et al.*, 1991; Weissman *et al.*, 1987) was sufficient to abolish transformation of tumor-derived human cell lines. In addition, using antisense RNA the possible transforming role of human papilloma virus proteins E6 and E7 has been further substantiated in cervical carcinoma cells and Hela cells (Von Knebel Doeberitz *et al.*, 1988). Furthermore it has been possible to demonstrate that inhibition of several proto-oncogenes (c-*myc*, c-*fos*, c-*myb*, c-*raf*) is able to block the growth of normal cells (Holt *et al.*, 1988; Nishikura and Murray, 1987; Gewirtz and Calabretta, 1988; Kolch *et al.*, 1991). Few studies, instead, have focused on the direct, stable inhibition of transformation by oncogenes (Amini *et al.*, 1986; Anfossi *et al.*, 1989; Becker *et al.*, 1989) or on the inhibition of growth factor receptors in human cancer cells.

To directly investigate the role of EGF-R overexpression in human tumors, we have tried to decrease EGF-R expression

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§ Supported by an EEC biotechnology fellowship and a fellowship from Fondazione L. Di Capua.

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[1] The abbreviations used are: EGF, epidermal growth factor; TGFα, transforming growth factor α; NCS, newborn calf serum; BMV, brome mosaic virus; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ab, antibody; mAb, monoclonal antibody.

in the human carcinoma KB cell line. In this paper we report that it is possible to reduce EGF-R expression to levels found in normal nontransformed cells, using EGF-R antisense RNA. Expression of antisense EGF-R RNA results in a suppression of the transformed phenotype of KB cells and restores serum and anchorage-dependent growth. In addition we show that the transformed phenotype is reduced proportionately to the decrease in expression of EGF-R, suggesting a primary role for the EGF-R in maintaining the tumoral state.

MATERIALS AND METHODS

Antisense EGF-R RNA Expression Vectors—The EGF-R cDNA fragment (*XbaI-HindIII*) derived from pSP ER7 (Clark *et al.*, 1986) was cloned in the pGEM 3 vector (pGEM ER7) in order to have the cDNA sense strand transcribed by the T7 polymerase and the antisense (AS) strand by the SP6 polymerase. pGEM ER-5 was constructed by subcloning the 5′ portion of the EGF-R cDNA (*XbaI-SmaI*) and pGEM ER3 by subcloning the 3′ EGF-R cDNA (*HincII-HindIII*). The human β-globin gene (pSP64 HBΔ6IVS,1–2) was kindly provided by Dr. T. Maniatis (Harvard University) (Krainer *et al.*, 1984) and subcloned in *PstI* site of the pGEM-4 vector (pGEM hβ-G).

The pact-CAT plasmid was kindly provided by Dr. S. Ishii (RIKEN, Japan) (Nishina *et al.*, 1989). The CAT insert was removed, and the 4.1-kb fragment of the EGF-R cDNA (*XbaI-HindIII*), the 0.89 kb 5′ fragment (*XbaI-SmaI*), and the 0.56 kb 3′ fragment (*HincI-HindIII*) were cloned by linker insertion in the opposite orientation to the β-actin promoter (pact-ER1, pact-ER5, and pact-ER3, respectively). For control transfections, the human β-globin gene was cloned by linker insertion in the same vector, in the antisense orientation (pact hβ-G).

In Vitro Transcription and Translation—To synthesize EGF-R mRNA, pGEM ER7 was linearized with *HindIII*. For synthesis of the antisense RNAs, pGEM ER7 was linearized with *XbaI* (AS-FL), *NaeI* (AS-K), or *HincII* (AS-3′); pGEM ER 5 was linearized with *XbaI* (AS-5′). pGEM hβ-G was linearized with *HindIII*. Templates were then transcribed according to the manufacturer (Promega) Sp6, T7 polymerase and BMV RNA were from Promega. Samples treated as described previously (Clark *et al.*, 1986) were further purified by $LiCl_2$ precipitation (2 M). RNAs (1 μg) were routinely run on 1% formaldehyde/agarose gel to determine the correct size and stored at −80 °C.

Cell Cultures, Transfection, and Agar Growth—KB cells, from American Type Tissue Culture, were maintained in Dulbecco's modified Eagle's medium, glutamine, penicillin, and streptomycin with 10% NCS. Tranfections were performed by calcium phosphate coprecipitation of the various plasmids with pSV2 neo (Gorman *et al.*, 1983), and cells were selected with 0.8 mg/ml of G418. Control transfections included cotransfection of the β-globin in the antisense orientation (pact hβ-G) and of the pact CAT vector alone with pSV2 neo; after selection these cells were used as control transfected cells.

For growth rate determination, cells were plated at 4×10^4 cells/60-mm dishes in regular medium. Eight hours later, cells were washed and plated in defined medium or in the same medium containing 0.5 or 10% NCS. The defined medium was Dulbecco's modified Eagle's medium/RPMI 1640 (50:50) supplemented with 3 μg/ml insulin, 5 μg/ml transferrin, and 4×10^{-8} M hydrocortisone (Amano *et al.*, 1988). Cells from duplicate dishes were changed and counted every 2nd day.

Colony formation efficiency was assessed by plating 400 cells/60-mm dishes in regular medium with 10% NCS. Ten days later, colonies were scored after fixing and Giemsa staining.

For growth in agar, 5500 cells/60-mm dishes were plated in agar with regular medium and 10% NCS as described previously (Velu *et al.*, 1989). Colonies were scored and photographed 2 weeks later.

^{125}I-EGF Binding and Immunoprecipitation—To screen for EGF-R expression, 2×10^5 cells/well were plated in 24-well dishes. Twenty-four hours later, ^{125}I-EGF binding was measured in triplicate wells as described (Beguinot *et al.*, 1984; Helin *et al.*, 1991). Scatchard analysis was performed as described (Helin *et al.*, 1991). Nonspecific binding was determined by excess cold EGF (1 μg/well) and was <5% of total.

For immunoprecipitation studies 2×10^6 cells/60-mm dishes were plated in regular medium and 8 h later incubated in methionine-free Dulbecco's modified Eagle's medium with 5% fetal calf serum containing 0.5 mCi of [^{35}S]methionine for 16 h. Cell extracts were then prepared (Beguinot *et al.*, 1984). Equal amounts of [^{35}S]methionine labeled extracts, as determined by trichloroacetic acid precipitation (Beguinot *et al.*, 1984), were immunoprecipitated with a polyclonal anti-EGF-R Ab (2913 Ab) (Beguinot *et al.*, 1986) or monoclonal anti-transferrin R (Oncogene Science) or monoclonal anti p34 Ab, followed by *Staphyloccoccus* A (the Enzyme Center) as described (Beguinot *et al.*, 1984). Immunoprecipitates were analyzed by SDS-PAGE. After fluorography, autoradiography was performed. Immunoprecipitates were quantified both by scanning of the autoradiogram exposed in the linear range and by direct counting of the radioactivity in the solubilized bands (Beguinot *et al.*, 1984) in a β counter.

RNA Preparation and Northern Blotting—Total RNA was extracted by the guanidine isothiocyanate procedure (Chomczynski and Sacchi, 1987) from 80% confluent 150-mm dishes.

For Northern blotting, 16 μg of total RNA was separated on 1% formaldehyde-agarose gels and transferred to nylon membranes (GeneScreen). ^{32}P-Labeled RNA probes were used to detect EGF-R mRNA and antisense RNAs. To detect the AS-FL and AS-3′ the pGEM ER3 was linearized with *HindIII* and transcribed by T7 polymerase; for the AS-5′ the pGEM ER5 was linearized with *PstI* and transcribed by T7 polymerase. To detect the EGF-R mRNA the pGEM ER3 was linearized with *HincII* and transcribed by the SP6 polymerase. ^{32}P-Labeled DNA probes to detect β-actin were prepared by random priming (Boehringer) of gel-purified cDNA. Prehybridization and hybridization were performed according to the manufacturer (GeneScreen). Blots were then washed at 65 °C at high stringency (Maniatis *et al.*, 1989) and exposed at −80 °C.

Microinjection—5×10^4 KB cells were plated in 35-mm polylysine (2 mg/ml)-coated dishes 48 h before microinjection. Cells were then washed and incubated with 100 nM EGF for 60 min at 37 °C to allow internalization and degradation of the receptor (Beguinot *et al.*, 1984). Cultures were washed, incubated in regular medium containing 10% serum, and cells microinjected with the various AS-RNAs together with affinipure rabbit IgG (1 mg/ml) in 2 mM Hepes, pH 7.6, 140 mM KCl. Immediately prior to injection, both the RNAs and the antibody solutions were centrifuged in a Beckman airfuge for 5 min at 25 p.s.i. In a typical experiment almost all the cells in the field (150–200 cells) were microinjected in 30 min. Microinjection was performed as described previously (Wehland *et al.*, 1981).

After injection, monolayers were rinsed with warm complete medium and incubated for 6 h at 37 °C to allow new EGF-R synthesis and reappearance on the cell surface. To improve visualization of the EGF-R, microinjected dishes were incubated again with 100 nM EGF for 30 min at 37 °C to induce EGF-R internalization (Beguinot *et al.*, 1984) and then processed for immunofluorescence.

Routinely, after microinjection, 1 μg of injected RNA was analyzed by agarose gel electrophoresis to ensure that it was not degraded during the procedure.

Immunofluorescence Microscopy—Cells were washed, fixed with 3.7% formaldehyde, and permeabilized with 0.1% Triton (Beguinot *et al.*, 1984). To detect the injected rabbit Ab, dishes were incubated with fluorescein-labeled goat anti-rabbit IgG for 15 min (50 μg/ml in phosphate-buffered saline, 0.1% bovine serum albumin, 0.1% saponin). To detect the EGF-R, dishes were then rinsed five times and sequentially incubated for 15 min with an anti-EGF-R antibody (EGFR-1, Amersham Corp.), washed, and followed by rhodamine-labeled rabbit anti-mouse IgG for 15 min (50 mg/ml in phosphate-buffered saline, 0.1% saponin, and 2 mg/ml normal goat IgG).

Cells were fixed and processed as described (Beguinot *et al.*, 1984). Cells were then observed with a Zeiss RA microscope equipped with rhodamine and fluorescein epifluorescence optics and photographed using Kodak tri-X film.

RESULTS

Cell-free Inhibition of EGF-R Translation by Antisense RNA—Sense and antisense RNA were prepared by introducing an EGF-R cDNA clone, or portions of it, into the pGEM-3 vector and synthesizing the RNA *in vitro* using the T7 polymerase (sense) or the SP6 polymerase (antisense). Four different antisense RNA molecules were engineered which encompassed the entire coding region (AS-FL), the kinase domain (AS-K), the 3′-coding region (AS-3′), or the 5′-coding and untranslated region (AS-5′), respectively (Fig. 1).

The antisense RNA species were first evaluated for their ability to inhibit the translation of the sense RNA strand *in vitro*. Each antisense RNA was mixed together with the full-length sense RNA in molar ratios of 1:1, 10:1, and 100:1,

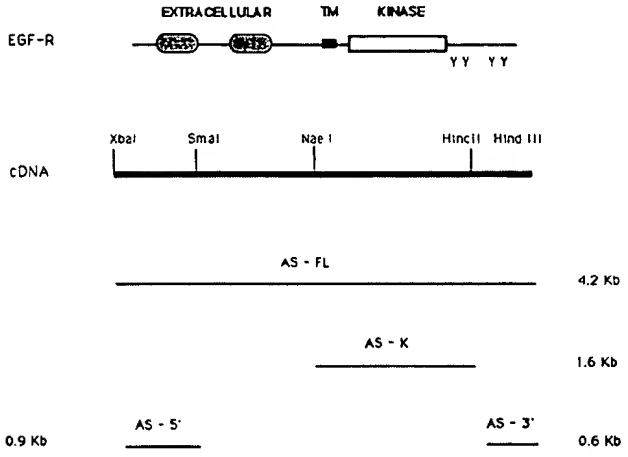
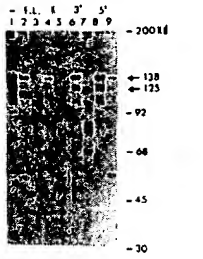


FIG. 1. **EGF-R antisense RNA.** Schematic representation of the hEGF-R cDNA drawn with the restriction sites utilized to subclone the antisense DNA constructs in the pGEM and pact expression vectors. AS-FL encodes the entire coding region plus approximately 150 base pairs of 5′- and 3′- untranslated region of the hEGF-R cDNA. AS-K includes mainly the kinase domain, AS-5′, the 5′-untranslated and coding region, and AS-3′ the 3′ end of the coding region. The EGF-R protein is drawn with the extracellular Cys-rich regions (*stippled oval*), the transmembrane domain (*TM*), the kinase domain (▭), and the C-terminal autophosphorylation sites, Tyr-1068, -1086, -1148, and -1173 (*Y*).

A



B

Inhibition of in vitro translation by EGF-R anti-sense RNA
(% residual activity)

	EGF-R RNA			BMV RNA	
	1	10	100	1	10
	(fold excess anti-sense/sense)				
-	100	100	100	100	100
AS-FL	47	5	n.d.*	127	120
AS-K	50	7	n.d.*	96	101
AS-3'	61	16	n.d.*	102	106
AS-5'	91	43	n.d.*	94	102

FIG. 2. ***In vitro* inhibition of EGF-R RNA translation by antisense RNAs.** EGF-R sense RNA and various antisense RNA were synthesized *in vitro* as described. *Left panel, in vitro* translation of the EGF-R mRNA was performed in a reticulocyte lysate system. Each of the EGF-R antisense RNAs was coincubated with the sense message at a molar ratio of 1:1 (*lanes 2, 4, 6,* and *8*) or 10:1 (*lanes 3, 5, 7,* and *9*) and analyzed by SDS-PAGE on a 7.5–15% polyacrylamide gel. *Lane 1,* no addition; *lanes 2* and *3,* AS-full-length; *lanes 4* and *5,* AS kinase; *lanes 6* and *7,* AS-3′; and *lanes 8* and *9,* AS-5′. *Arrows* indicate the *in vitro* made EGF-R protein. Exposure of the autoradiogram was for 12 h. *Right panel,* after autoradiography, EGF-R protein band and control brome mosaic virus protein bands were excised and solubilized and their [^{35}S]methionine content determined. Results are expressed as percentage of the total activity when no antisense RNA was added to the *in vitro* translation system. Results are the average of two (*BMV*) or three (*EGF-R*) independent experiments. *n.d.* = not detectable, <1%.

respectively, added to a reticulocyte lysate translation system and the relative amount of translated EGF receptor determined. As observed previously, *in vitro* translation of EGF-R RNA resulted in a major band of 138 kDa, corresponding to the full-length protein, and a 125-kDa band, which is probably derived from premature termination (Fig. 2 and Clark *et al.*, 1986). Each antisense RNA was inhibiting EGF receptor synthesis almost completely when present at a 10-fold higher concentration (Fig. 2). At this ratio, the AS-5′ RNA, complementary to the 5′ end of the coding region, was the least effective *in vitro,* and it required a higher concentration to completely block translation of the sense mRNA. To control for nonspecific effects, antisense RNAs were mixed with BMV RNA (molar ratio 1:1 and 10:1) and then incubated in the cell-free system. EGF-R antisense RNAs failed to inhibit the translation of BMV. They were also unable to inhibit *in vitro* translation of total poly(A$^+$) RNA extracted from KB cells or translation of the human globin RNA. In addition, globin antisense RNA was unable to block *in vitro* translation of EGF-R RNA (data not shown). Therefore the effects of the antisense RNA were specific for the EGF-R mRNA.

Transient Inhibition of EGF-R Synthesis by Microinjection of Antisense RNA—To determine if the antisense EGF-R RNAs could inhibit translation of the EGF-R mRNA in cells, the *in vitro* synthesized antisense RNAs were microinjected into the cytoplasm of KB cells which had been transiently depleted of pre-existing EGF-R by EGF-induced down-regulation (Beguinot *et al.*, 1984). The ability of the microinjected cells to synthesize new EGF receptor protein was then followed by immunofluorescence. This procedure allows the rapid identification of all injected cells (fluorescein-labeled diffuse pattern), as well as the visualization of the residual EGF receptor levels (rhodamine-labeled, punctuate pattern). To enhance receptor visualization, cells were also treated shortly with EGF to induce EGF-R accumulation in endocytic vesicles (Beguinot *et al.*, 1984). Fig. 3 shows the result of a typical experiment in which either no RNA (*A*), an antisense EGF-R RNA (*B*), or a control antisense globin RNA (*C*) were injected in KB cells. The fluorescein-positive cells, identified by fluorescein staining of the coinjected antibody (Fig. 3, *A″*, *B″*, and *C″*) were also stained with rhodamine-labeled anti-EGF-R antibodies to determine the extent of expression of newly synthesized EGF-R (Fig. 3, *A′*, *B′*, and *C′*). Control cells injected with antibody alone showed the typical punctuate pattern due to internalized EGF-R (*A′*). A similar pattern was also observed in the cells injected with globin antisense RNA, indicating no significant inhibition of EGF-R synthesis (*C′*). Only the cells injected with an antisense EGF-R RNA showed a dramatic decrease of the EGF-R fluorescent signal, suggesting that the antisense RNA was able to efficiently block EGF-R translation.

All the EGF-R antisense constructs were tested in this manner for their ability to block EGF-R synthesis *in vivo.* KB cells were injected with various antisense RNAs and scored for presence of receptor six hours after the injection. As shown in Fig. 4*A*, all the antisense RNAs were able to inhibit EGF-receptor synthesis. The antisense complementary to the 3′-coding region of the EGF-R mRNA (AS-3′) was the most effective: more than 90% of the injected cells were completely inhibited. The full-length antisense RNA (AS-FL) was also effective (58% negative cells), whereas the antisense RNA complementary to the kinase or 5′ regions (AS-K and AS-5′) were less efficient in blocking EGF-R synthesis (28 and 40%, respectively). When globin antisense RNA was injected as control, less than 10% of the cells were clearly negative for expression suggesting that the inhibitory effect was largely specific. Concentration-dependent inhibition of EGF-R synthesis by the 3′ antisense RNA was observed (Fig. 4*B*). Injection of as few as 700 molecules/cell produced a significant inhibition of EGF receptor synthesis and the inhibition was almost complete with larger amounts of antisense RNA.

Stable Expression of Antisense RNA in KB Cells—The antisense constructs complementary to the entire coding region (AS-FL) or to the 3′ and 5′ end of the EGF-R mRNA (AS-3′, AS-5′) were cloned downstream from the β-actin

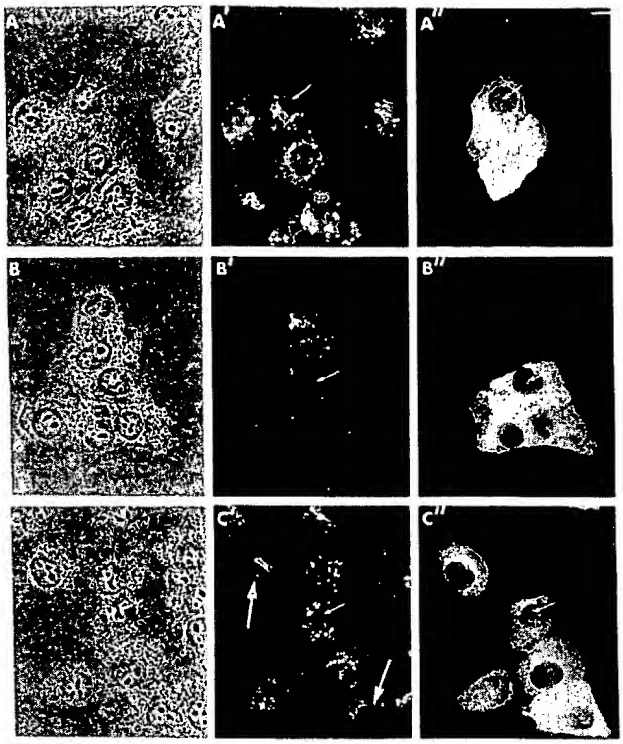


FIG. 3. **Inhibition of translation of EGF-R mRNA in KB cells by microinjection of EGF-R antisense RNA.** KB cells were incubated with 100 nM EGF for 1 h at 37 °C, washed, and microinjected with affinity-pure rabbit IgG (1 mg/ml) alone (*A*) or together with AS-3′ (*B*) or globin AS-RNA both at a concentration of 1 mg/ml. Cells were incubated for 6 h at 37 °C, and 100 nM EGF was then added for 30 min more. Dishes were fixed and permeabilized, incubated sequentially with fluorescein-labeled anti-rabbit IgG and then with anti EGF-R mAb (EGFR-1) followed by rhodamine-labeled anti-mouse IgG. *A–C* show a phase contrast picture of the cells in the field; A′,B′, and C′ show the localization of the EGF-R in the injected cells, detected by rhodamine anti-mouse IgG, and *A″*, *B″*, and *C″* show the localization of the injected rabbit antibody, detected by fluorescein anti-rabbit IgG. *Small arrows* indicate examples of injected cells in which normal amounts of EGF-R (*A′* and *C′*) or no EGF-R (*B′*) were detected. *Big arrows* indicate injected cells which show an intermediate amount of EGF-R. (Magnification ×567: *bar*, 10 μm.)

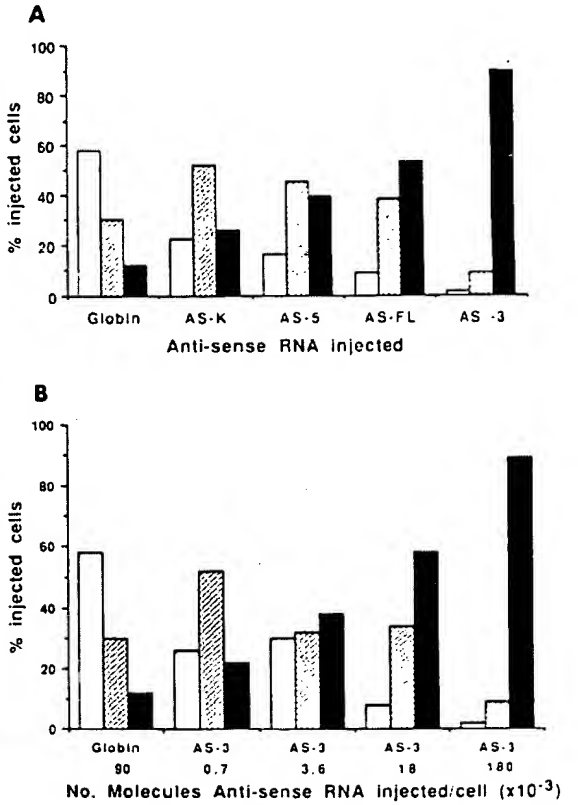


FIG. 4. **Inhibition of EGF-R mRNA translation by microinjected antisense RNA: effect of the various EGF-R AS RNA and concentration dependence.** *A*, KB cells were treated and microinjected as described previously with EGF-R antisense RNAs AS-FL, AS-K, AS-5′, AS-3′, or with globin antisense RNA, each at 1 mg/ml, together with 1 mg/ml rabbit IgG. Injected cells were identified, counted, and then analyzed for EGF-R fluorescence. The amount of EGF-R present was quantified on the base of fluorescence brightness and number of positive vesicles observed compared to noninjected cells. Cells were categorized as positive, if expressing a normal amount of receptors (*open bars*), negative, if almost no signal was detected (*closed bars*), and intermediate, if the amount was reduced but still detectable (*hatched bars*). Results were expressed as a percentage of positive, negative, or intermediate cells over total number of cells injected. At least 150 cells were counted in each experiment. Values represent the mean of five (AS-3′ and globin AS) or three independent experiments (AS-FL, AS-5′, AS-K). *B*, KB cells were microinjected with rabbit IgG and AS-3′ RNA at a concentration of 1 mg/ml (180×10^3 molecules/cell), 100 μg/ml (18×10^3 molecules/cell), 20 μg/ml (3.6×10^3 molecules/cell), 4 μg/ml (0.7×10^3 molecules/cell), or 1 mg/ml globin AS RNA (90×10^3 molecules/cell). Injected cells were counted and the amount of EGF-R present quantified. Results were expressed as percentage of cells that scored positive (*open bars*), negative (*closed bars*), or intermediate (*hatched bars*) over the total number of cells. Results are derived from five (1 mg/ml globin AS and AS-3′, as presented in *A*) or two independent experiments (lower concentration of AS-3′).

promoter and transfected in KB cells. Stable clones were selected by G-418 resistance and 103 clones were then screened by ^{125}I-EGF binding to measure the residual level of EGF receptor. Approximately 60% of the clones showed a small reduction (20–30%) of ^{125}I-EGF binding and were not further analyzed. Twenty-one clones showed a consistently greater reduction of ^{125}I-EGF binding (ranging from 50 to 88%), suggesting an inhibition of EGF-R synthesis by the antisense RNA (Table I). Control transfections were performed with the vector alone or with a plasmid encoding β-globin antisense RNA (pact hβ-G). Fifteen of these control clones were tested by ^{125}I-EGF binding and were found to bind EGF similarly to parental KB cells, indicating no effect on EGF-R synthesis (Table I and Fig. 5). These clones were used as additional control cells in all successive experiments.

The proportion of inhibited clones was similar with all three antisense constructs suggesting that they were all effectively inhibiting EGF-R synthesis. We have, however, consistently observed a lower transfection efficiency with the full-length antisense DNA (AS-FL) in four independent transfections (from 3- up to 20-fold lower). This finding may suggest either a more effective inhibition of this construct in blocking EGF-R synthesis or perhaps a toxic effect due to the length of the RNA duplex.

Biochemical Characterization of KB Cells Expressing Antisense RNA—To determine the relative amount of EGF-R synthesized in antisense expressing clones, cells were metabolically labeled with [^{35}S]methionine, and the EGF-R was immunoprecipitated with an anti-EGF-R polyclonal antibody (2913 Ab). Fig. 5 shows the low residual levels of EGF-R in several clones transfected with the different antisense RNA constructs. Quantitation of the immunoprecipitated receptor correlated well with the reduced ^{125}I-EGF binding capacities of the clones, indicating that the residual receptors were

TABLE I

EGF-R distribution in KB clones transfected with EGF-R antisense RNA

G-418-resistant clones obtained after transfection of control or anti-sense DNA plasmids were tested by ^{125}I-EGF binding as described under "Materials and Methods." Clones transfected with control DNAs had a standard deviation from parental KB cells in ^{125}I-EGF binding of <10%.

	No. of clones	Clones reduced in EGF-R	
		>20%	>50%
Control			
pact CAT	10	0	0
pact hβ-G	6	0	0
Antisense			
AS-FL	35	22	6
AS-5′	36	18	7
AS-3′	32	18	8
Total	103	58	21

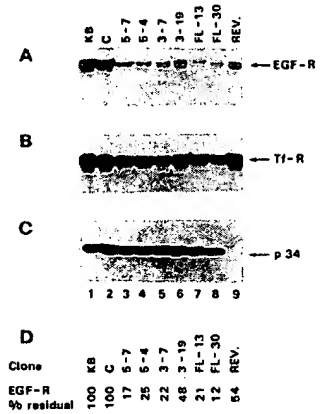


FIG. 5. **Immunoprecipitation of the EGF-R in antisense expressing KB cells.** Equal amounts of [^{35}S]methionine-labeled cell extracts from KB and antisense transfected cells (2×10^7 cpm) were immunoprecipitated with an anti EGF-R polyclonal Ab (*A*), an anti-transferrin receptor monoclonal Ab (*B*), or a monoclonal Ab against a soluble protein p34 (*C*) and analyzed by SDS-PAGE on a 8.5% polyacrylamide gel. *Lane 1*, parental KB cells; *lane 2*, mock transfected KB cells; *lane 3*, AS 5-7; *lane 4*, AS 5-4; *lane 5*, AS 3-7; *lane 6*, AS 3-19; *lane 7*, AS FL-13; *lane 8*, AS FL-30; *lane 9*, revertant (*Rev*) derived from AS 5-7. Exposures of the autoradiograms were 30, 16, and 4 h, respectively. *D*, the amount of EGF-R in the immunoprecipitated bands was quantitated both by densitometric scanning of the autoradiograms and by directly counting the bands excised and eluted from the gels. Results are expressed as percentage of residual EGF-R compared with parental KB cells and are the average of two or three independent experiments.

properly processed and exported to the cell surface. In agreement with previous reports (Beguinot *et al.*, 1985; Lyall *et al.*, 1987), KB cells expressing 2×10^5 EGF-R/cell show one class of receptors with an affinity of 1.3 nM (Fig. 6). As shown in Fig. 6, clone as 5-7 shows an 80% reduction in total receptor number with no significant change in the affinity for EGF (K_d 0.65 nM). Similar results were obtained with another very reduced clone (FL-30) and an intermediate clone (AS3-19) (data not shown), further suggesting no biochemical alteration of residual EGF-Rs. To ensure that EGF-R antisense RNA selectively reduced expression of the EGF-R, cell lysates from control and antisense expressing cells were immunoprecipitated with antibodies against another membrane receptor, the transferrin receptor, and against a soluble protein p34. Both proteins are quite abundant in KB cells. As shown in Fig. 5, *B* and *C*, no significant change in the level of expression of

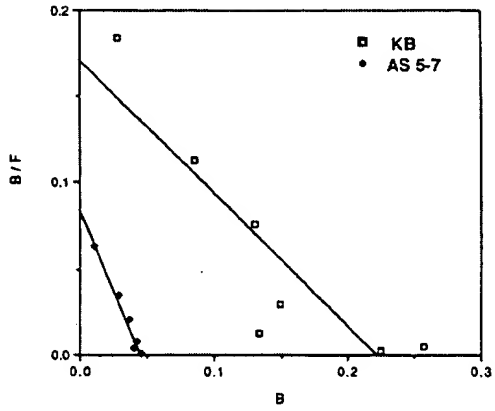


FIG. 6. **Scatchard analysis of ^{125}I-EGF binding to intact KB and antisense expressing cells.** Confluent AS 5-7 (○) and parental KB cells (□) in 24-well Costar dishes were incubated for 2 h on ice with different concentrations of ^{125}I-EGF as described under "Materials and Methods." Each value represents the mean of triplicate determinations. Binding results were plotted as Scatchard analysis and fitted to one site model to determine number of receptors and affinity for the ligand. Bound (*B*), in nanomolar.

p34, and only a limited clearance of the transferrin receptor were detected in antisense expressing cells compared with untransfected or control transfected cells.

Northern blots were performed using RNA probes to antisense RNAs in clones exhibiting reduced levels of EGF receptors. As shown in Fig. 7, antisense RNA complementary to the 5′ or 3′ region was expressed at relatively high levels in the clones (*A* and *B*, *top*), whereas the AS-FL RNA was detected in lower amounts (*C*, *top*). The antisense DNA constructs were integrated in high copy number in the genome, as determined by Southern analysis (data not shown), and a difference in copy number could explain the different level of expression observed in the various clones.

In addition the amount of the EGF-R mRNA expressed at steady state in the clones was reduced compared with parental or control transfected KB cells (Fig. 7*D*, *top*). Quantitation of the autoradiograms indicated a good correlation between total amount of antisense RNA expressed and the degree of reduction of EGF-R mRNA and protein in the various clones.

As control for the specificity of inhibition by the antisense RNA, parallel blots were hybridized with a human β-actin probe. No significant differences in β-actin mRNA expression were observed between control and antisense expressing cells (*A–D*, *bottom*), indicating that the EGF-R anti-sense RNA was not aspecifically inhibiting RNA translation.

Biological and Transforming Properties of Antisense Expressing Cells—In examining the growth properties of several antisense expressing cells, we noticed that these cells had lower growth rates compared with parental or control transfected cells. Some clones, such as AS 5-7 and AS FL-30, reached a much lower density (3-fold lower than control cells) in medium with 10% serum (Fig. 8). Doubling time, calculated during the exponential growth phase, was significantly increased in the above mentioned clones (36 *versus* 22 h for parental cells). There were also morphological differences between cells expressing low levels of EGF-R and parental cells: cells appeared more elongated and fibroblastic-like and more adherent to the substrate (data not shown).

KB cells, like most transformed cells, are able to grow in medium devoid of growth factors as well as in medium supplemented with serum (Fig. 8). However, KB clones with low residual level of EGF-Rs were unable to grow in defined medium (Fig. 8) or in medium with low serum (data not

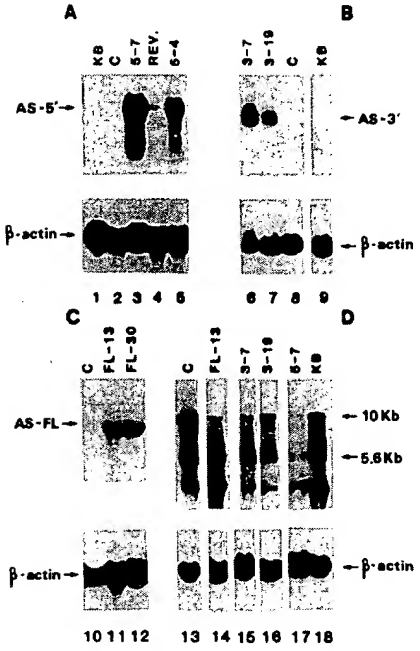


FIG. 7. **Expression of antisense RNA and EGF-R mRNA in antisense KB cells.** Total RNA was isolated from parental and antisense transfected KB cells. Sixteen μg of total RNA were run on agarose gel, blotted on nylon filters, and hybridized with specific ^{32}P-labeled riboprobes for detection of the 5′ (*A, top*), 3′ (*B, top*), full-length (*C, top*) antisense RNAs or for EGF-R mRNA (*D, top*). The *bottom part* of each panel shows parallel blots hybridized with a random primed ^{32}P-labeled DNA probe specific for the human β-actin mRNA. In each panel, *arrows* indicate the relevant RNA bands. *A: lane 1,* KB cells; *lane 2,* control transfected cells (*C*); *lane 3,* AS 5–7; *lane 4,* revertant cells derived from AS 5-7 clone; *lane 5,* AS 5-4. *B: lane 6,* AS 3-7; *lane 7,* AS 3-19; *lane 8,* control transfected cells (*C*); *lane 9,* KB cells. *C: lane 10,* control transfected cells; *lane 11,* AS FL 13; *lane 12,* AS FL 30. *D: lane 13,* control transfected cells (*C*); *lane 14,* AS FL 13; *lane 15,* AS 3-7; *lane 16,* AS 3-19; *lane 17,* AS 5-7; *lane 18,* KB cells.

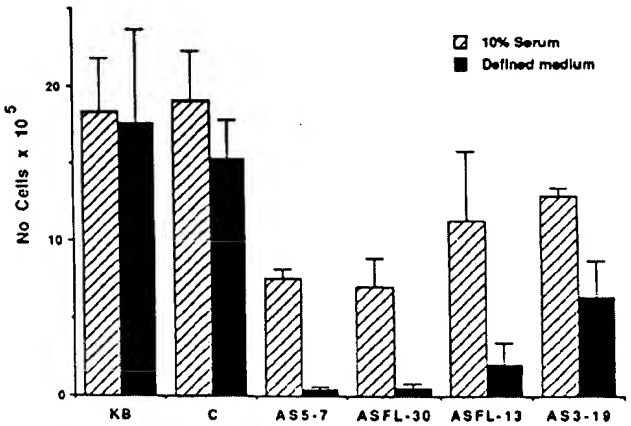


FIG. 8. **Growth in serum-free medium of KB cells expressing EGF-R antisense RNA.** 5 × 10^4 parental, control, and antisense transfected KB cells were plated in 60-mm dishes in regular medium. Eight hours later they were changed into defined medium or in medium supplemented with 10% NCS. Cells were counted 8 days after plating. Results presented are the average of two or three independent experiments.

shown). An intermediate behavior was observed in cells expressing approximately 50% of EGF-R compared with parental cells. Therefore, by reducing expression of EGF-R in the transfected cells, a more normal phenotype, that of serum-dependent growth was restored.

In addition we analyzed antisense clones on the basis of the two other transformation parameters for cells in culture: colony formation under sparse culture condition and ability to grow in soft agar. Parental or control transfected cells had a viability of more than 90% in forming colonies or in growing in semisolid medium (Table II). The most inhibited clones had a much lower ability of forming colonies or growing in agar. An intermediate phenotype was observed in cells expressing intermediate levels of receptors (Table II and Fig. 9). Although the clones with very reduced EGF-R expression were still partially able to grow in agar, more than 70% of the

TABLE II

Biological properties of antisense expressing KB cells

	EGF-R	Doubling time[a]	Colony formation[b]		Soft agar colonies[c]	
			%	Cells/dish	%	Colonies/dish
	% wild type	*h*				
KB	100	22	100	360	100	5000 ± 600
C	100	23	103	370	98	4900 ± 90
AS FL-30	12	36	27	100	26	1320 ± 240
AS FL-13	21	NT[d]	33	129	NT[d]	
AS 5-7	15	36	27	100	33	1650 ± 230
AS 5-4	50	25	71	250	61	3050 ± 300
AS 3-7	50	NT[d]	72	260	52	2600 ± 300
AS 3-19	48	25	67	240	42	2320 ± 300
REV 5-7	89	22	110	390	95	4700 ± 320

[a] Cells were plated at 5 × 10^4/60-mm dish in regular medium supplemented with 10% NCS and counted every second day in duplicate. The doubling time was calculated during the exponential phase of growth. Results are the average of at least three independent experiments. By the Student's *t* test the difference between parental and AS 5-7 and AS FL-30 was significant ($p < 0.05$).

[b] Four-hundred cells were plated in 60-mm dishes in triplicate with regular medium supplemented with 10% NCS. Ten days later, colonies were fixed, stained, and counted. Results are the average of two to three independent experiments.

[c] Cells were plated in soft agar at 5.5 × 10^3/dish in regular medium supplemented with 10% NCS. Total number of colonies (big and small) were scored 15 days later. Results are the average of three independent experiments.

[d] NT, not tested.

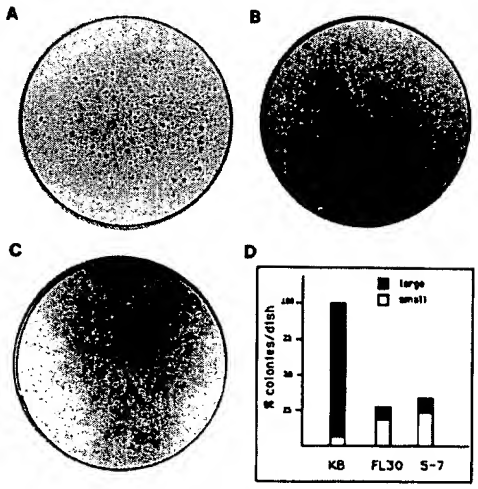


FIG. 9. **Growth in agar of KB cells expressing EGF-R antisense RNA.** 5.5 × 10^3 KB (*A*) or antisense expressing cells, AS FL-30 (*B*), and AS 5-7 (*C*), were plated in soft agar with medium supplemented with 10% NCS and grown for 15 days. Colonies were then counted and divided in small (<60 μm) or large (>250 μm). *D* indicates the size distribution of the colonies for KB, AS FL-30, and AS 5-7 cells. *Hatched bars,* large colonies; *open bars,* small colonies.

colonies were very small as compared with parental clones (Fig. 9*D*). In addition most of the small colonies formed by antisense transfected cells did not grow even if kept in culture for a month (data not shown). Clones expressing intermediate levels of EGF-R tended to form colonies of intermediate size. These results suggest that the transforming phenotype is directly dependent on the levels of EGF-R in KB cells.

Taken together these data indicate that growth of the antisense clones was severely impaired. For this reason, the most reduced clones had a clear tendency to revert to a less severe phenotype. During this study we isolated several spontaneous revertants, and the growth properties of one of these, derived from clone AS 5–7, are presented in Table II. The revertant clone had reacquired expression of elevated EGF-R levels and showed growth and transforming properties similar to wild type cells. Although this clone was still G-418 resistant, it had lost many copies of the antisense DNA, as determined by Southern analysis (data not shown), and was expressing less than 10% as many antisense RNA molecules compared with the AS 5–7 clones (Fig. 7). Similar results were observed with other revertants (data not shown). This finding is suggestive of an essential primary role of the EGF-R in KB cells in culture.

DISCUSSION

Several lines of evidence suggest an oncogenic role for the EGF receptor in the development of human squamous carcinoma and glioblastoma. Although overexpression of EGF-R in rodent cells has clearly demonstrated that the activated EGF-R is able to induce transformation, the only approach to understand its role in human malignancies is to inhibit specifically its overexpression in human cancer cells and study their consequent phenotype. In the case of proteins essential for growth, however, it may prove impossible to drastically reduce their levels without affecting cell viability. In order to test the feasibility of an antisense approach for the EGF-R, we first tested if the *in vitro* synthesized antisense RNA could effectively inhibit translation of the EGF-R mRNA in the test tube. We then injected the antisense RNAs into KB cells to study the short term inhibition of EGF-R synthesis.

We have utilized the human carcinoma KB cell line since many parameters of EGF-R biosynthesis and degradation have been extensively characterized in these cells. KB cells express about 200,000 EGF-R, show no amplification of the gene (Xu *et al.*, 1984), are frankly tumorigenic in culture and in nude mice, and do not respond mitogenically to EGF. KB cells are known to produce TGFα mRNA (Derynck *et al.*, 1987), but the presence of TGFα in the medium or the possibility of a growth stimulatory autocrine loop has not been further addressed (for review see Derynck, 1988). Early responses to EGF, however, are easily detectable in these cells. In response to EGF, the EGF receptor is rapidly degraded, with a half-life of 1 h (Beguinot *et al.*, 1984). This subsequently induces EGF-R mRNA and protein synthesis, which both peak 4 h after EGF addition (Clark *et al.*, 1985). The newly synthesized EGF-R can be initially localized in the Golgi region by immunofluorescence and then, after 6 h approximately 70% of total receptors have reached the plasma membrane.[2]

Taking advantage of these properties we have devised a protocol to totally deplete the cells of pre-existing EGF-R, by a short pretreatment with EGF, and we have studied the effect of the antisense RNA in inhibiting *de novo* synthesis of EGF-R. All EGF-R antisense constructs were effective in blocking mRNA translation, both after microinjection into KB cells and *in vitro*. Surprisingly, however, the least effective construct was the one complementary to the 5′-untranslated and coding region of EGF-R mRNA. This finding is in contrast with some reports showing that the 5′ antisense RNA is the most effective in blocking message translation (Izant and Weintraub, 1985; Melton, 1985).

The 5′ region of the EGF-R mRNA has a potential stem and loop structure which includes the translation initiation codon (Merlino *et al.*, 1985). This secondary structure could be responsible for the relative inefficient pairing between mRNA and 5′ antisense RNA. Moreover other groups have shown that oligonucleotides complementary to the 3′-coding region or to the splice site junctions of several RNA can be quite effective in blocking their translation (Ch'ng *et al.*, 1989). Our results further demonstrate the difficulty in defining general rules for antisense targeting and the utility of testing constructs complementary to several portions of the mRNA.

While *in vitro* antisense RNAs were able to inhibit EGF-R RNA translation even at a molar ratio of 1:1 (50% inhibition), *in vivo* the concentration of the RNA required to exert a good inhibitory effect was much higher (100–1000-fold). We have estimated that KB cells express about 50 molecules of EGF-R mRNA at steady state. Therefore the lowest concentration of antisense RNA injected (700 molecules) was at least 10-fold in excess and was just slightly able to block EGF-R synthesis. It is possible that the rate of degradation of naked RNA in the cytoplasm is very fast or the endogenous mRNA may be protected by ribosomes and therefore less accessible for hybridization. Microinjection into the nucleus did not significantly improve the efficacy of the antisense RNA (data not shown). However it prompted us to use a strong constitutive promoter to drive expression of EGF-R antisense RNA in long term transfection experiments. We, thus, selected the β-actin promoter because is very active in KB cells[3] and has proven useful in promoting expression of antisense RNAs in other cell systems (Gunning *et al.*, 1987).

The screening of more than 100 transfected clones suggested that all three antisense constructs (complementary to the 5′-, 3′-, and full-length coding region of the mRNA) were equally effective, since the number of significantly inhibited clones (more than 50% reduction in EGF-R) was approximately the same. However we also noted a consistently lower transfection efficiency (3–20-fold lower) with the longer antisense RNA (AS-FL), suggesting a toxic effect of this construct. We favor the possibility of a more effective block of EGF-R mRNA translation so that the clones negative for EGF-R expression would not grow. Alternative possibilities could be the length of the putative mRNA/antisense RNA duplex or the inability of degradation of the duplex.

In studying the growth properties of the antisense transfected KB cells, we noticed that the cells were growing with reduced rates even in medium supplemented with serum. The doubling time of some clones with reduced EGF-R levels (12–17%) was 36 h compared with 22 h in the parental cells and the saturation density was 3-fold lower. Clones expressing intermediate levels of EGF-R (50% of control) exhibited an intermediate behavior. These results suggest that the EGF-R is essential for the proliferation of KB cells and may explain our inability to obtain clones with no EGF-R expression.

In medium devoid of growth factors, clones expressing only 25,000–40,000 receptors/cell (AS FL-30, AS 5-7,) were completely unable to grow; intermediate clones grew at lower density in comparison with parental cells. Therefore, a net

[2] L. Beguinot and M. C. Willingham, unpublished observation.

[3] S. Ishii, personal communication.

decrease in EGF-R expression to levels comparable with more normal cells, such as human fibroblasts or keratinocytes, induces a totally restored serum-dependent growth and abolishes one of the transformation parameters for cells in culture. Growth was partially re-established in these antisense expressing cells by the addition of EGF to the cultures, whereas no effect of EGF was detected for parental cells.[4] We would be tempted to speculate that by drastically decreasing EGF-R expression, the proposed autocrine loop which supports growth of KB cells is abolished. Experiments are in progress to study the parameters of TGFα production and exogenous EGF stimulation in antisense expressing cells.

Ability of colony formation and growth in agar were also clearly reduced in the most inhibited clones. In agar the few colonies produced by the antisense expressing clones grew as very small colonies, whose size did not further increase even if kept in culture for more than 1 month. This finding suggests that these cells could sustain only a limited number of divisions in agar even if supplemented with serum. Antisense clones expressing 50% of total receptors had an intermediate phenotype: they were able to form medium size colonies in agar and were only partially able to grow in medium without growth factors. Taken together, although these data do not demonstrate that the EGF-R is the only oncogene in KB cells, they show a good correlation between levels of EGF-R and transformation parameters and suggest that the transformed phenotype of KB cells may depend on overexpression of the EGF-receptor. By extension all the secondary events, which may have taken place during the transformation of these cells, may be largely controlled by EGF-R activation, since a restored normal phenotype can be achieved by blocking EGF-R expression. Although the minimal amount of EGF-R sufficient to induce transformation has never been determined, in NIH 3T3 cells, we have previously shown that overexpression of normal EGF-R to levels comparable with parental KB cells is sufficient to induce transformation in the presence of the ligand (Velu *et al.*, 1989a, 1989b; Helin *et al.*, 1991) and that transformation is then proportional to the degree of EGF-R overexpression. In addition EGF-R overexpression induces transformation in Rat-1 cells which express approximately 30,000 endogenous receptors (*i.e.* levels similar to some of the antisense expressing clones) (Haley *et al.*, 1989), although a proper quantitation of EGF-R receptors is not reported in this study.

To the best of our knowledge this is the first report in which a stable inhibition of a growth factor receptor is sufficient to block growth and transformation of a human cancer cell line. Our experiments also show the feasibility of such an approach in cells directly explanted from human tumors.

Finally our results support the possible therapeutic effectiveness of reagents, such as blocking antibodies (Mendehlson, 1988; Aboud-Pirak *et al.*, 1988), toxin-conjugated ligand (Chaudary *et al.*, 1987), or tyrosine kinase-specific inhibitors (Yaish *et al.*, 1988) to inhibit specifically the growth of EGF-R-overproducing human tumors.

Acknowledgments—We thank Dr. Ira Pastan for many discussions and continual encouragement throughout this work, Drs. Douglas R. Lowy and Glenn T. Merlino for helpful discussions, David Olson for critical reading of the manuscript, Dr. S. Ishii for providing pact-CAT plasmid.

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